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Inducible Proteins in Citrus Rootstocks with Different Tolerance Towards the Root Rot Pathogen *Phytophthora palmivora*

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Abstract

Activities of defence-related proteins (β-1,3-glucanases, chitinases and peroxidases) and concentrations of total soluble phenolics were measured in roots and leaves of non-infected and infected plants to investigate the response of different citrus rootstock genotypes to the root rot pathogen *Phytophthora palmivora* Butler. Infection with the pathogen increased concentrations of total proteins, total phenolics and β-1,3-glucanase activity in roots of all genotypes, and increases were associated with the extent of root mass reductions and thus susceptibility of the plants. Root chitinase and root peroxidase levels were slightly reduced or unaltered upon infection. β-1,3-Glucanase activity was also elevated in leaves of infected plants, but increases did not differ between tolerant and susceptible rootstocks. Effects of root infection on leaves were typically the reverse of effects on roots for chitinase- and peroxidase levels and more pronounced in susceptible rootstock genotypes. Although differences in enzyme expression were observed between susceptible and tolerant citrus seedlings, effects were usually associated with disease progression, and not with resistance to *P. palmivora*. It is suggested that increased activities of the proteins and soluble phenolics studied are not implicated in the primary defence to *Phytophthora* root diseases, but may contribute to the inhibition of the pathogen during infection in tolerant citrus.

Introduction

Plants are continuously subjected to attack by insect herbivores, bacteria, viruses and fungi. Upon attack, plants often respond by induction of defence mechanisms including biochemical and structural changes (Agrios, 2005). Structural changes involve the strengthening of cell walls by depositing callosic and cellulosic material, a process often accompanied by the production of phenolic compounds. Some phenolic compounds accumulate in response to pathogen infection and play an important role in the active defence response of the host (Nicholson and Hammerschmidt, 1992; Osbourne, 1996; De Ascensao and Dubery, 2003). Other phenolics are part of the constitutive defences of healthy plant tissues and are associated with non-host resistance.

Biochemical changes associated with defence responses include the synthesis of phytoalexins (Hammerschmidt, 1999) and pathogenesis-related (PR) proteins (Van Loon, 1997; Datta and Muthukrishnan, 1999). Pathogenesis-related proteins are defined as plant proteins induced in pathological situations, such as infection with microbial organisms or attack by phytophagous insects, or by application of chemicals inducing host-responses (Van Loon et al., 1994). However, these proteins can be induced by other stresses and have often been detected in healthy tissues of many plants. The term inducible defence-related proteins distinguishes PR proteins, which are not detectable in healthy non-infected tissue, from other inducible plant proteins which are constitutive but increasingly induced by pathogens (Van Loon et al., 2006).

Among the PR proteins most intensively studied in plant-fungal interactions are β-1,3-glucanases and chitinases. β-1,3-Glucanases are involved in physiological and developmental processes in healthy plants, including cell division, embryogenesis, fruit ripening and seed germination (Leubner-Metzger and Meins, 1999). They catalyse the hydrolytic cleavage of 1,3-β-D-glucosidic linkages in β-1,3-glucans, components not only of plant cell walls, but also of the cell wall of fungal pathogens. Hence, these enzymes have been of major interest in the study of plant-fungal interactions and their possible use as biochemical markers for disease resistance has been demonstrated (Anguelova et al., 1999; Kini et al., 2000; Shetty et al., 2001).

Chitinases hydrolyse the β-1,4-linkage between *N*-acetylglucosamine residues of chitin, a cell-wall polysaccharide in many fungi and the exoskeleton of invertebrates (Neuhaus, 1999). β-1,3-Glucanases and

chitinases are capable of degrading fungal cell-wall components *in vivo* and *in vitro*, resulting in the inhibition of fungal growth (Abeles et al., 1971; Schlumbaum et al., 1986; Mauch et al., 1988; Kim and Hwang, 1997). The cell walls of oomycetes such as *Phytophthora* spp. predominantly consist of $(1 \rightarrow 3)$ and $(1 \rightarrow 6)$ linked β-glucans and contain no or only small amounts of chitin (Bartnicki-Garcia, 1968; Ruiz-Herrera, 1992). However, studies showed that in many plant-pathogen systems the combined action of glucanase and chitinase is required for optimal antifungal activity (Mauch et al., 1988; Sela-Buurlage et al., 1993; Jongedijk et al., 1995; Ji and Kić, 1996). Apart from acting directly by degrading fungal cell walls, β-1,3-glucanases and chitinases may also contribute indirectly to host-resistance by releasing oligosaccharide products from the cell walls of the invading pathogen which act as elicitors of plant defence responses (Boller, 1995).

Peroxidases occur in numerous isoforms in plants and are implicated in a wide range of physiological processes (Gaspar et al., 1982; Hiraga et al., 2000). As they are involved in the formation of reactive oxygen species and are key enzymes of cell wall-building processes, which include phenol oxidation and lignification, their role in the enhancement of disease resistance has been addressed in numerous studies (Shetty et al., 2001; Nawar and Kuti, 2003; Shivakumar et al., 2003).

In Florida, *Phytophthora* diseases of citrus cause economic losses from damping-off of seedlings in nurseries, foot rot of the trunk and fibrous root rot in groves leading to yield losses and tree decline (Timmer and Menge, 1988; Graham and Menge, 1999). The most important *Phytophthora* spp. affecting citrus worldwide are *Phytophthora nicotianae* Breda de Haan (syn. *Phytophthora parasitica* Dastur), *Phytophthora citrophthora* Smith & Smith and *Phytophthora palmivora* Butler. Although *P. nicotianae* has long been regarded as the main cause for root diseases in Florida, recent years have shown that *P. palmivora* is a far more aggressive and competitive pathogen and that many rootstock genotypes not affected by *P. nicotianae* are highly susceptible to this species (Graham, 1995; Bowman et al., 2002). *Phytophthora citrophthora* Smith & Smith causes brown rot of fruit and gummosis on tree trunks particularly in Mediterranean climates, but is usually not a serious problem in Florida (Graham et al., 1998).

Using resistant rootstocks is one of the most successful strategies to manage *Phytophthora* diseases, and their development and selection is one of the primary objectives of the USDA citrus breeding programmes in Florida (Bowman et al., 2002, 2003; Grosser et al., 2003; Albrecht and Bowman, 2004). Valuable information for resistance breeding may be gained by understanding the host response to the pathogen at the biochemical level. Despite the abundance of literature on plant-pathogen interactions, few studies focus on diseases occurring below-ground (Benhamou et al., 1990; Burketová et al., 2003) or on the differential expression of enzyme activities in different plant tissues (Beerhues and Kombrink, 1994; Kini et al., 2000; Borowicz et al., 2003). The objective of this study was to determine if fungal disease resistance is associated with biochemical changes in roots and leaves of citrus, and if PR proteins are useful as biochemical markers of resistance towards the root rot pathogen *P. palmivora*.

Materials and Methods

The plant material was from a previous study which evaluated citrus rootstocks in response to *Phytophthora* root infection (Albrecht and Bowman, 2004).

Rootstock genotypes

Seven rootstock genotypes were used for all analyses: Cleopatra mandarin (*Citrus reticulata* Blanco), Sun Chu Cha (*C. reticulata*), Swingle citrumelo [*C. grandis* (L.) Osbeck × *Poncirus trifoliata* (L.) Raf.], US-897 (*C. reticulata* 'Cleopatra' × *P. trifoliata*), Carrizo citrange [*C. sinensis* (L.) Osbeck × *P. trifoliata*], Pineapple sweet orange (*C. sinensis*) and sour orange (*C. aurantium* L.).

Experimental set-up

Six, 63 cm × 40 cm × 22 cm plastic tubs were filled to a depth of 10 cm with potting mix composed of steam-sterilized peat/perlite/vermiculite (Pro-Mix BX, Premier Horticulture Inc., Red Hill, PA, USA). For infected treatments, citrus roots were collected from the soil under the canopy of trees at a field site determined by soil dilution plating to be heavily infected with *P. palmivora*. Three tubs were inoculated by layering 360 g of the washed citrus roots on top of the potting mix and adding additional potting mix to a final depth of 18 cm. For non-infected treatments, an equal mass of citrus roots from greenhouse-grown trees of the same genotype was added to the remaining three tubs. Molecular and serological analyses performed before the onset of the experiment established that roots obtained from the field were infected with *P. palmivora* in contrast to the roots obtained from greenhouse-grown trees, which did not contain any *Phytophthora* or other related pathogenic organisms (Albrecht and Bowman, 2004).

Three, 13-week-old seedlings of each rootstock genotype were transplanted into the tubs from cone pots and arranged randomly for a total of 21 seedlings per tub. Tubs were placed into plastic trays and arranged in three randomized blocks along the greenhouse bench. Plants were grown under natural light conditions and potting mix was kept to near field capacity by maintaining the water at a level of 2–3 cm above the bottom of the tub. Plants were fertilized by addition of a water-soluble fertilizer mix, 20N-10P-20K (Peters Professional, The Scotts Company, Marysville, OH, USA) at a rate of 500 mg^{-1} l^{-1} N once every week. Four weeks after initiating the experiment, plants were removed from the potting mix. Roots and leaves were washed thoroughly with tap water, blotted

dry, ground in liquid nitrogen with a mortar and pestle and stored at −80°C until used for immunological and biochemical assays.

Prior to the main experiment, a preliminary study was conducted using 14-week-old seedlings from the rootstock genotypes Sun Chu Sha, US-897, Carrizo citrange and Swingle citrumelo. Experimental procedures were as described for the main experiment; however, a fine-loamy, poorly drained soil of the Winder type, sometimes used for citrus production in Florida, was used as a growing medium. Soil was allowed to drain freely and kept near field capacity by watering every other day. Plants were arranged in a completely randomized manner using five seedlings per rootstock genotype and treatment. Seven weeks after initiating the experiment roots were collected and used for biochemical assays.

Enzyme-linked immunosorbent assays (ELISA)

The amount of pathogen present in the roots was measured with a multiwell-test system (Agdia Incorporated, Elkhart, IN, USA), employing a polyclonal anti-*Phytophthora* antibody and a monoclonal alkaline phosphatase-conjugated secondary antibody. Immunological assays were performed according to the manufacturer's instructions using 100 mg root tissue/ml extraction buffer. All assays were performed in duplicate.

Protein extraction

Approximately 800 mg of root or leaf tissue from non-infected and infected plants was suspended in 16 ml ice-cold 0.1 M sodium phosphate buffer (pH 7.4) containing 0.48 g of hydrated polyvinylpyrrolidone (PVPP; Sigma, St. Louis, MO, USA) and mixed overnight at 4°C. Samples were centrifuged at 20 000 ***g*** for 15 min and supernatants were filtered through one layer of Miracloth (Calbiochem, La Jolla, CA, USA) into dialysis tubing (molecular cutoff of 6–8 kDa, Spectrum, Laguna Hills, CA, USA). Extracts were dialysed overnight against dH_2O at 4°C and lyophilized. The lyophilized material was resuspended in 1.2 ml of dH_2O and centrifuged for 10 min at 10 000 ***g***. Supernatants were used for protein and enzyme analyses.

Protein assays

Total protein was measured according to Bradford (1976), using bovine serum albumin (fraction V) as the standard.

Phenolic analysis

Total soluble phenolics were extracted from 100 mg of plant tissue in 1 ml 70% acetone for 30 min at RT by vortexing the tissue in 5 min intervals. After centrifugation at 20 000 ***g*** for 5 min, supernatants were used for analyses. Phenolic concentrations were determined according to Waterman and Mole (1994) using Folin-Ciocalteu reagent (Sigma) with tannic acid as the standard and expressed as mg tannic acid equivalents/g tissue.

Enzyme assays

Peroxidase activities were measured as described in Worthington (1993). Reactions were conducted in 0.1 M potassium phosphate buffer (pH 7.0) for 3 min at RT using 4-aminoantipyrine as the hydrogen donor. Enzyme activities were expressed as $\Delta A_{510}\ min^{-1}\ mg^{-1}$ protein. β-1,3-Glucanase activities were determined by measuring the production of reducing sugars from laminarin (*Laminaria digitata*, Sigma) using glucose (Glc) as the standard. Reactions were conducted in 0.1 M sodium acetate buffer (pH 5.0) for 20 min at 50°C. Total reducing sugars were measured at 540 nm by the colorimetric method of Nelson (1944) using the reagents of Somogyi (1952), and enzyme activities were expressed as μmol Glc $min^{-1}\ mg^{-1}$ protein. Chitinase activities were measured according to Wirth and Wolf (1990) using soluble dye-labelled chitin (CM-Chitin-RBV, Loewe Biochemica, Munich, Germany) as the substrate. Reactions were conducted in 0.2 M sodium acetate buffer (pH 5.0) for 10 min at 37°C and enzyme activities were expressed as $\Delta A_{550}\ min^{-1}$ mg protein. All biochemical assays were performed at least in duplicate.

Statistical analyses

All data were subjected to analysis of variance (ANOVA) employing a split-plot model with block as a random factor and infection and rootstock genotype and their interaction as fixed experimental factors. Individual data for the three seedlings per rootstock genotype were averaged within each sub-plot unit. Significant ANOVA tests were followed by multiple comparisons of means using Tukey's HSD procedure. Data from the preliminary study were analysed employing a completely randomized model with infection and rootstock genotype and their interaction as fixed experimental factors. All analyses were performed with STATISTICA version 6.0 (StatSoft, Tulsa, OK, USA).

Results

Performance of rootstock genotypes analysed in the preliminary experiment appeared to be influenced strongly by the field soil used for this study, which is unfavourable for some citrus rootstocks. The presence of other unidentified microorganisms in the soil may have contributed to this effect. However, results for total protein content and enzyme activities measured in roots of non-infected and infected plants were similar to the results of the main study and are summarized here.

In the preliminary study, infection significantly decreased root mass by 35% on average ($F_{1,32} = 31.336$, $P < 0.001$). Infection significantly affected total protein concentrations ($F_{1,32} = 40.224$, $P < 0.001$), peroxidase activity ($F_{1,32} = 15.355$, $P < 0.001$) and β-1,3-glucanase activity ($F_{1,32} = 25.860$, $P < 0.001$). No interaction of infection and genotype was observed. Infection of roots increased the amount of total proteins in all rootstock genotypes by 92% on average. Peroxidase activity was reduced

by 22% on average and β-1,3-glucanase activity was increased by up to 458%. A small increase of β-1,3-glucanase levels (13%) was observed for Sun Chu Sha, but constitutive enzyme levels were significantly higher compared with the other rootstock genotypes. Chitinase activity was not significantly affected by infection.

In the following results of the main experiment are presented in detail.

Root reductions and pathogen levels

Growth data for non-infected and infected seedlings, including shoot mass and shoot length were discussed in Albrecht and Bowman (2004). For the present study only data for root mass and ELISA are summarized.

Root mass varied among rootstock genotypes ($F_{6,24} = 20.646$, $P < 0.0001$) and was significantly decreased by infection ($F_{1,24} = 148.606$, $P < 0.01$), but the extent of this decrease varied among genotypes ($F_{6,24} = 12.799$, $P < 0.001$; Fig. 1). Root mass reduction was lowest in Cleopatra and Sun Chu Sha (21–25%), moderate in US-897 and Sour orange (32–36%) and highest in Pineapple, Carrizo and Swingle (56–61%; Fig. 1). Based on the extent of root mass reductions, rootstock genotypes were categorized as tolerant (Cleopatra and Sun Chu Sha), moderately tolerant (US-897 and Sour orange) and susceptible (Pineapple, Carrizo and Swingle) towards *P. palmivora*. No significant effect for block as a random factor was observed ($F_{2,24} = 1.587$, $P > 0.05$).

Molecular analyses of roots using PCR–RFLP techniques confirmed that all plants inoculated with field roots were infected with *P. palmivora* (Albrecht and Bowman, 2004). Enzyme-linked immunosorbent assays performed to quantify the amount of pathogen in the root tissue revealed significant differences ($F_{6,14} = 12.656$, $P < 0.0001$) among rootstock genotypes (Fig. 2). Lowest absorbance values of 0.46–0.78 were observed in roots of Sun Chu Sha, Sour orange and Cleopatra, and highest values between 0.91 and 1.21 were obtained for Pineapple, Swingle, Carrizo and US-897. Absorbance values for non-infected plants were zero.

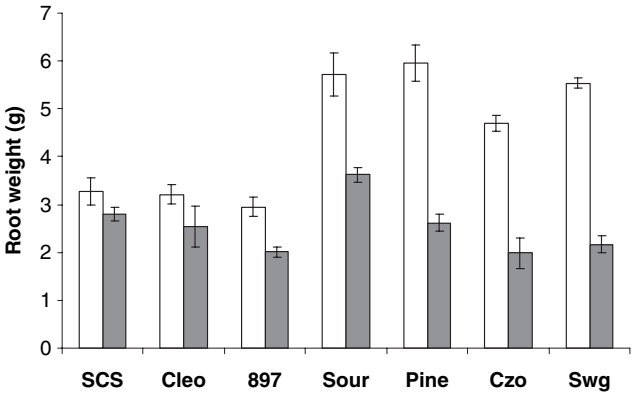

Fig. 1 Average root mass of non-infected (□) and infected (■) citrus seedlings 4 weeks after inoculation with *Phytophthora palmivora*. SCS, Sun Chu Sha; Cleo, Cleopatra mandarin; 897, US-897; Sour, Sour orange; Pine, 'Pineapple' sweet orange; Czo, Carrizo citrange; Swg, Swingle citrumelo. Vertical bars represent standard errors. (Albrecht and Bowman, 2004; modified)

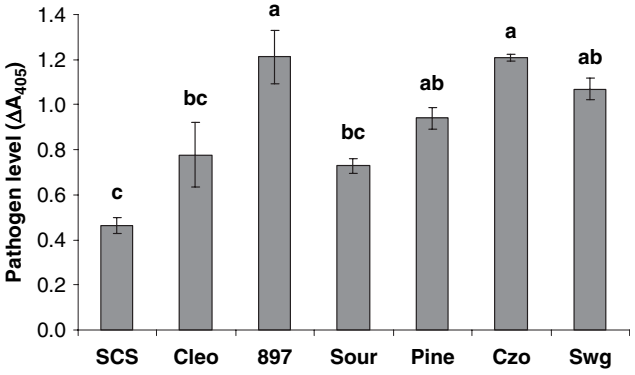

Fig. 2 Pathogen levels of roots from citrus seedlings infected with *Phytophthora palmivora* measured using enzyme-linked immunosorbent assays 4 weeks after inoculation. Mean values are expressed as absorbances at 405 nm. Vertical bars represent standard errors. SCS, Sun Chu Sha; Cleo, Cleopatra mandarin; 897, US-897; Sour, Sour orange; Pine, 'Pineapple' sweet orange; Czo, Carrizo citrange; Swg, Swingle citrumelo. Different letters above bars indicate significant differences between means according to Tukey's HSD test ($P < 0.05$). (Albrecht and Bowman, 2004; modified)

Root proteins, phenolics and enzymes

Infection and genotype significantly affected all variables (Table 1). Interaction of infection and genotype was significant for protein, phenolics and β-1,3-glucanase. A significant effect for block as a random factor was observed only for peroxidase.

Phytophthora palmivora infection increased the amount of total proteins in all rootstock genotypes (Fig. 3a). Protein concentrations were increased by 76% on average, with smallest increases (25–38%) observed in roots from the tolerant genotypes Sun Chu Sha and Cleopatra. The largest increases (94–125%) were observed in the susceptible rootstock genotypes Pineapple, Carrizo and Swingle, which exhibited the largest root mass reductions. Constitutive protein levels were highest in Sun Chu Sha and Cleopatra, whereas induced concentrations were highest in all susceptible genotypes. Except for US-897, protein concentrations of roots from infected plants were higher in plants exhibiting the highest pathogen levels.

Similar results were observed for total phenolics concentrations (Fig. 3b), which were increased by 62% on average in infected roots, with the largest increases (81–91%) observed in roots of susceptible Carrizo, Swingle and Pineapple. Constitutive phenolic levels were highest in Sun Chu Sha and Swingle, while induced levels were highest in the susceptible genotypes.

Infection of roots with *P. palmivora* reduced peroxidase concentrations by 15% on average (Fig. 3c). Constitutive and induced levels of peroxidase in roots did not vary between tolerant and susceptible rootstock

Table 1
ANOVA results for total protein, total soluble phenolics, peroxidase-, β-1,3-glucanase- and chitinase activities in roots and leaves from citrus seedlings of seven different rootstock genotypes non-infected or infected with *Phytophthora palmivora*

	Roots			Leaves		
Effect	Df	F	P	Df	F	P
Protein						
Block (B)	2	4.722	ns	2	22.160	*
Infection (I)	1	1885.865	***	1	0.829	ns
B × I	2	0.104	ns	2	0.334	ns
Genotype (G)	6	3.672	**	6	4.837	**
I × G	6	5.939	***	6	1.032	ns
Phenolics						
Block (B)	2	0.720	ns	2	18.655	ns
Infection (I)	1	93.287	*	1	13.676	ns
B × I	2	8.649	**	2	2.195	ns
Genotype (G)	6	23.347	***	6	15.274	***
I × G	6	21.051	***	6	1.241	ns
Peroxidases						
Block (B)	2	154.977	**	2	53.360	*
Infection (I)	1	168.556	**	1	20.105	*
B × I	2	0.097	ns	2	0.360	ns
Genotype (G)	6	4.453	**	6	7.483	***
I × G	6	1.880	ns	6	7.791	***
β-1,3-Glucanase						
Block (B)	2	0.849	ns	2	41.378	*
Infection (I)	1	26.520	*	1	466.261	**
B × I	2	5.074	*	2	0.120	ns
Genotype (G)	6	29.620	***	6	7.429	***
I × G	6	2.552	*	6	0.803	ns
Chitinases						
Block (B)	2	1.846	ns	2	31.039	*
Infection (I)	1	40.053	*	1	66.159	*
B × I	2	0.205	ns	2	0.519	ns
Genotype (G)	6	5.134	**	6	4.270	**
I × G	6	1.166	ns	6	3.967	**

ns, $P > 0.05$; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

genotypes and no relation of enzyme levels to concentrations of soluble phenolics was observed.

β-1,3-Glucanase activities in roots of infected plants were increased across all rootstock genotypes and varied significantly within treatments (Fig. 3d). Enzyme activities were increased by 168% on average, and smallest increases (87–111%) were measured in Sour orange, Pineapple, Sun Shu Sha and Cleopatra. Susceptible rootstocks Carrizo and Swingle exhibited increases of up to 220%, whereas β-1,3-glucanase activity in roots of US-897, which was classified as moderately tolerant towards *P. palmivora*, was increased by 380%. With the exception of Pineapple, genotypes with highest pathogen levels detected in the root tissue thus exhibited the largest increases in β-1,3-glucanase concentrations. Constitutive enzyme levels were highest in Sour orange and Pineapple whereas induced levels were highest in susceptible rootstock genotypes and in Sour orange.

Like peroxidases, activities of root chitinases were reduced in most rootstock genotypes upon infection with *P. palmivora* (Fig. 3e). Enzyme reductions were 10% on average and largest (17–19%) in roots of Sour orange and Carrizo. Chitinase concentrations did not differ between tolerant and susceptible plants and were not related to β-1,3-glucanase activities.

Leaf proteins, phenolics and enzymes

Infection significantly affected peroxidases, β-1,3-glucanase and chitinases (Table 1). Genotype significantly affected all variables, whereas interaction of both main effects was significant for peroxidases and chitinases only. A significant effect for block as a random factor was observed in all variables but phenolics.

Infection of roots with *P. palmivora* did not significantly alter levels of total proteins and total phenolics in citrus leaves (Table 1) and no differences within non-infected or infected rootstock genotypes were observed (Fig. 4a,b).

Contrary to observations for root tissue, which exhibited small but consistent reductions in peroxidase levels with infection, peroxidase activities in leaves were increased by 39–65% in Pineapple, Carrizo and US-897 and by 188% in Swingle (Fig. 4c). These rootstock genotypes exhibited the highest levels of *Phytophthora* as measured with ELISA. Enzyme activities of Cleopatra, Sun Chu Sha and Sour Orange, which exhibited lowest pathogen levels, were reduced by 13–27%upon infection.

Similar to β-1,3-glucanase activities measured in roots, leaf β-1,3-glucanase concentrations were increased in infected citrus seedlings (Fig. 4d). However, the average increase in enzyme levels was twofold lower than the mean increase observed in infected roots and did not differ considerably among genotypes. Similar to roots, induced leaf β-1,3-glucanase concentrations were somewhat higher in susceptible rootstock genotypes and in Sour orange. Constitutive enzyme levels did not vary between genotypes.

Infection of roots with *P. palmivora* increased chitinase activities in citrus leaves (Fig. 4e), contrary to chitinase levels measured in roots, which were decreased upon infection. Increase in leaf chitinase levels was 39% on average, with largest increases observed in susceptible genotypes Pineapple and Swingle. Constitutive enzyme levels did not vary between genotypes.

Discussion

Pathogenesis-related proteins have been the subject of numerous studies regarding their involvement in host-resistance towards pathogens, and their potential as biochemical markers for breeding programmes has been demonstrated (Sariah et al., 2001; Nawar and Kuti, 2003; Shivakumar et al., 2003). In this study, the expression of peroxidases, β-1,3-glucanases, chitinases and total soluble phenolics in citrus seedlings was examined and their usefulness as markers for resistance to *Phytophthora* diseases evaluated.

Inoculation of citrus seedlings with the root rot pathogen *P. palmivora* significantly reduced the root mass of plants from all seven rootstock genotypes analysed. Based on the extent of root mass reductions, the mandarin rootstocks Cleopatra and Sun Chu Sha were categorized as tolerant towards *P. palmivora*, whereas Pineapple sweet orange and the trifoliate rootstocks Carrizo citrange and Swingle citrumelo proved to be

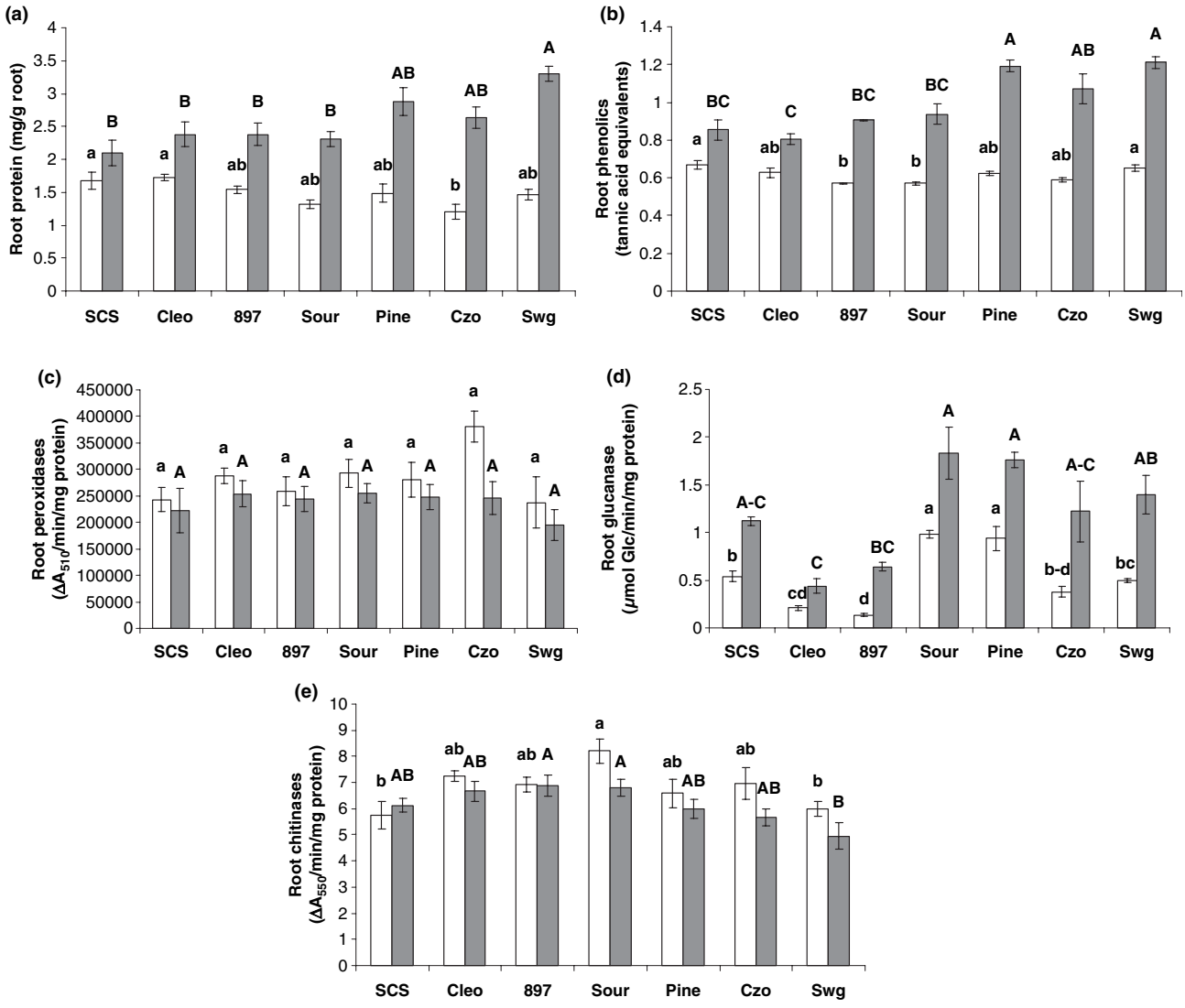

Fig. 3 Comparison of (a) total protein, (b) total soluble phenolics, (c) peroxidase activity, (d) β-1,3-glucanase activity and (e) chitinase activity in roots of non-infected (□) and infected (■) citrus seedlings 4 weeks after inoculation with *Phytophthora palmivora*. Means ± SE are presented. SCS, Sun Chu Sha; Cleo, Cleopatra mandarin; 897, US-897; Sour, Sour orange; Pine, 'Pineapple' sweet orange; Czo, Carrizo citrange; Swg, Swingle citrumelo. Different letters above bars indicate significant differences between means according to Tukey's HSD test ($P < 0.05$) for non-infected roots across rootstocks (lower case letters) and for infected roots across rootstocks (upper case letters)

susceptible (Albrecht and Bowman, 2004). US-897 (a mandarin × trifoliate hybrid) and Sour orange were classified as moderately tolerant towards this pathogen. The amount of pathogen in roots measured immunologically was generally associated with the degree of root mass reductions with the exception of US-897, which exhibited pathogen levels similar to or larger than the susceptible rootstocks (Albrecht and Bowman, 2004).

Infection with *P. palmivora* resulted in an increased concentration of total proteins in roots, particularly in susceptible genotypes. No significant changes of protein levels were observed in leaves.

Induced synthesis of phenolic compounds is common in many plant-pathogen interactions and often associated with host-resistance. In the present study, total soluble phenolics of roots were increased in all rootstock genotypes, but induced phenolic concentrations and their relative increase after infection were lowest in roots of tolerant citrus seedlings. Leaf phenolic levels were not changed upon infection and did not differ significantly between tolerant and susceptible plants. These results are in marked contrast to the findings of Sariah et al. (2001) and Thangavelu et al. (2003), who reported that higher constitutive and induced levels of phenolics, respectively, contributed to resistance of banana to *Fusarium oxysporum*. Similarly, Geetha et al. (2005) reported a larger concentration of soluble phenolics in root tissues of pearl millet cultivars resistant to downy mildew when compared with susceptible cultivars following infection. Yamunarani et al. (2004) suggested that elevated levels of phenolics and defence-related proteins may be involved in the suppression of early blight in tomato. Apparently, the

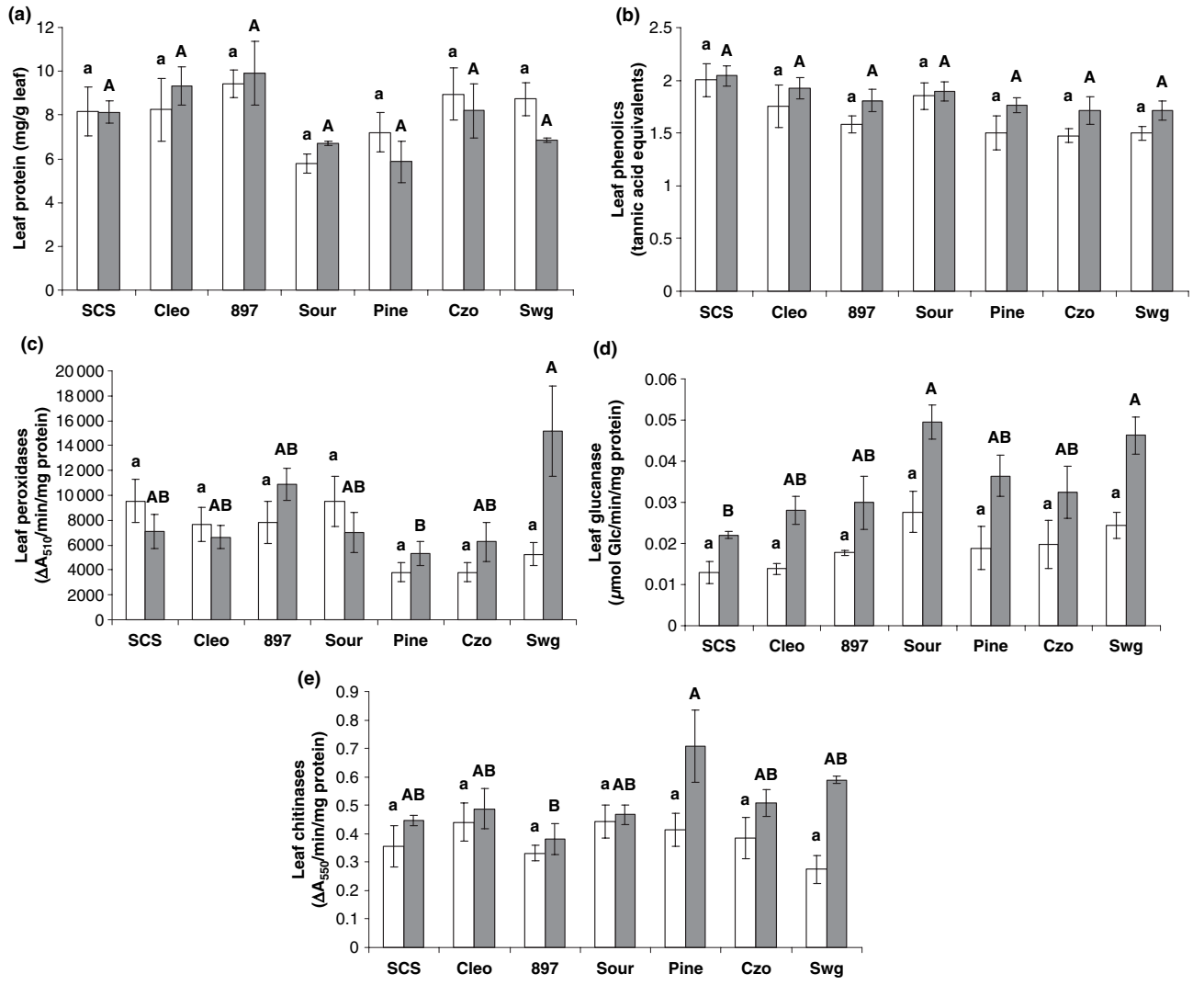

Fig. 4 Comparison of (a) total protein, (b) total soluble phenolics, (c) peroxidase activity, (d) β-1,3-glucanase activity and (e) chitinase activity in leaves of non-infected (□) and infected (■) citrus seedlings 4 weeks after inoculation with *Phytophthora palmivora*. Means ± SE are presented. SCS, Sun Chu Sha; Cleo, Cleopatra mandarin; 897, US-897; Sour, Sour orange; Pine, 'Pineapple' sweet orange; Czo, Carrizo citrange; Swg, Swingle citrumelo. Different letters above bars indicate significant differences between means according to Tukey's HSD test ($P < 0.05$) for non-infected roots across rootstocks (lower case letters) and for infected roots across rootstocks (upper case letters)

increased synthesis of soluble phenolics in roots of citrus seedlings observed in the present study is associated with pathogenesis and not with the effective containment of the pathogen at the site of infection. More studies are necessary to determine not only the concentration of phenolics, but also their nature, including wall-bound compounds, to determine if they play a role in *Phytophthora* resistance.

Increased synthesis of phenolics is often correlated with an increased activity of peroxidases, the enzymes associated with cell-wall formation and lignification (Graham and Graham, 1991; Deborah et al., 2001; Sariah et al., 2001). Infection of roots with *P. palmivora* decreased peroxidase levels in roots of tolerant and susceptible citrus seedlings and enzyme activities were not associated with levels of soluble phenolics. On the contrary, peroxidase activities were increased in leaves of the susceptible rootstocks and in leaves of US-897, whereas the remaining rootstocks exhibited slightly reduced peroxidase concentrations. Shivakumar et al. (2003) observed that peroxidase levels increased in resistant pearl millet seedlings, but decreased in susceptible cultivars upon infection with the downy-mildew pathogen *Sclerospora graminicola*. Also, constitutive peroxidase levels were clearly correlated with resistance, contrary to the findings of the present study. Constitutive and induced activities of peroxidase have also been associated with resistance in several other host-pathogen systems (Wyatt et al., 1991; Shetty et al., 2001; Nawar and Kuti, 2003; Thangavelu et al., 2003). The enzymes have since been used in numerous transgenic studies, but results were rather inconclusive. Overexpression of a peroxidase gene in transgenic potato did not increase resistance to *Fusarium* or *Phytophthora* (Ray et al., 1998); neither was resistance to fungal pathogens enhanced in transgenic

tomato plants (Lagrimini et al., 1993). Moreover, enhanced expression of the enzyme increased susceptibility of Norway spruce to *Phytophthora parasitica* var. *nicotianae* (Elfstrand et al., 2001a) and negatively influenced plant growth and plant development (Lagrimini et al., 1997; Elfstrand et al., 2001b). Similar to the observations in the present study, Ray and Hammerschmidt (1998) found that neither phenolic content nor peroxidase levels were critical to resistance of potato to the dry rot pathogen *Fusarium sambucinum*. The fact that peroxidases exist in a large number of isozymes in the plant and that they are involved in a broad range of physiological processes may be part of the reason why their role in plant resistance remains uncertain. Results of the present study suggest that peroxidases, like phenolics, are not directly implicated in the primary defence response of citrus to *P. palmivora*, although they may be involved in secondary processes connected with pathogenesis.

Due to their ability of degrading fungal cell walls, β-1,3-glucanases and chitinases have been extensively studied in many host-pathogen interactions and enzyme activities were typically correlated with disease resistance (Wyatt et al., 1991; Anguelova et al., 1999; Egea et al., 1999; Shetty et al., 2001; Tonón et al., 2002). In the present study, infection of citrus roots with *P. palmivora* resulted in a marked increase of β-1,3-glucanase concentrations in roots and leaves of all rootstock genotypes. By far the largest increase was observed in roots of US-897, followed by the susceptible rootstocks Carrizo and Swingle. Relative increases of β-1,3-glucanase activities in leaves were similar for all rootstock genotypes. Induced enzyme levels of roots and leaves were highest in susceptible genotypes and in one of the tolerant genotypes. These findings differ from the findings of Kini et al. (2000), who observed highest enzyme activities in highly resistant pearl millet cultivars upon downy mildew infection and thus suggested the possible use of β-1,3-glucanase as a biochemical marker for resistance screening. Similarly, the rapid accumulation of β-1,3-glucanase in pepper cultivars was shown to be associated with resistance to the pathogen *Phytophthora capsici* (Egea et al., 1999). Anguelova et al. (1999) demonstrated the association of leaf rust resistance in wheat with high constitutive levels of β-1,3-glucanase. On the contrary, constitutive enzyme levels were not correlated with disease resistance in the present study on citrus. However, the remarkable increase of β-1,3-glucanase activity in roots of US-897 observed here seems to be directly linked to pathogen levels, which were among the highest in this rootstock, despite the moderate root loss. Moreover, as US-897 is a hybrid derived from *P. palmivora*-tolerant and *P. palmivora*-susceptible parentage, increased β-1,3-glucanase activity may indeed suggest the involvement of this enzyme in host tolerance, although at a later stage in disease progression (Albrecht and Bowman, 2004). Whereas susceptible rootstocks of trifoliate parentage may reach a maximum enzyme level upon infection, tolerant mandarin genotypes may increase β-1,3-glucanase concentrations after infection to levels necessary for containment of the pathogen. Yet, it cannot be excluded that increased β-1,3-glucanase levels of roots were in fact the result of an increased secretion of the enzyme by the fungus and not by the host, as was observed for melons after infection with *Colletotrichum lagenarium* (Rabenantoandro et al., 1976).

β-1,3 Glucanases and chitinases have been shown to act synergistically in the degradation of fungal cell walls (Mauch et al., 1988; Jongedijk et al., 1995) and their combined expression may be important for the optimal function of defence responses. Infection of citrus roots with *P. palmivora* reduced root chitinase levels, and enzyme activities were similar in susceptible and tolerant rootstock genotypes. Chitinase concentrations were not linked to root glucanase concentrations. As oomycetes like *Phytophthora* do not possess much chitin in their cell walls, these observations are not unexpected and in accordance with Mauch et al. (1988), who observed that none of the oomycetes tested in their study were inhibited by chitinase, β-1,3-glucanase or a combination of these enzymes. However, chitinase levels were clearly elevated in leaves of susceptible citrus seedlings upon infection with *P. palmivora*, apparently the result of an induced systemic response occurring above-ground. Similar to the results presented here, Pegg and Young (1981) observed increased β-1,3-glucanase and chitinase activities in susceptible tomato plants after infection with *Verticillium*, which were correlated with the distribution and the amount of the fungus in the tissue. As observed for total soluble phenolics and peroxidases, it appears that chitinases and β-1,3-glucanase are not involved in the primary defence response of citrus to root rot caused by *P. palmivora*. Different results were observed in transgenic studies of plants constitutively expressing these enzymes. Masoud et al. (1996) demonstrated that transgenic alfalfa plants constitutively expressing both enzymes showed reduced disease symptoms after infection with *Phytophthora megasperma*, but not after infection with several chitin-containing pathogens. Likewise, combined expression of β-1,3-glucanases and chitinases resulted in increased fungal resistance in transgenic tobacco and tomato (Zhu et al., 1994; Jongedijk et al., 1995). Resistance of transgenic wheat plants to scab was increased under greenhouse conditions, but was not observed under field conditions (Anand et al., 2003). Overexpression of chitinase genes also lead to an improved tolerance in several host-pathogen systems (Grison et al., 1996; Terakawa et al., 1997; Tabaeizadeh et al., 1999), but constitutive glucanase overexpression in alfalfa did not enhance its tolerance to the anthracnose fungus *Colletotrichum trifolii* (Salles et al., 2002). These results, together with the results presented here, demonstrate that disease resistance is a complex process which not only involves multiple resistance genes, but which also depends on the host-pathogen system and on other developmentally and environmentally regulated factors.

In summary, none of the proteins examined in this study proved to be useful as a biochemical marker for *P. palmivora* resistance in citrus. Western blot-analyses of different isoforms of these proteins performed in this laboratory confirm this view (data not shown). Whether other isoforms not detected with the procedures used in our studies play a role in disease resistance to *Phytophthora* remains to be established. The results presented here lead to the conclusion that these enzymes are products of defence-related genes, which contribute to the inhibition of the pathogen when the plant expresses resistance, but do not determine the specificity of the interaction of *P. palmivora* and citrus. Tolerance to the pathogen may be achieved through the release of other defence-related proteins or antimicrobial compounds not examined in this study. The findings that leaf proteins, particularly peroxidases and chitinases, generally reflect pathogenesis occurring below-ground in a reversed manner above-ground, as observed in a previous study (Borowicz et al., 2003), may possibly prove useful for evaluating disease progression during the course of the experiment without the need for destructive sampling of valuable plant material.

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